#8

PATENT
0147-0189P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: Rainer FISCHER et al.

Appl. No.: 09/419,788 Group: 1643

Filed: October 18, 1999 Examiner: UNKNOWN

For: MOLECULAR PATHOGENICIDE MEDIATED PLANT DISEASE RESISTANCE

L E T T E R

Assistant Commissioner for Patents
Washington, DC 20231

May 24, 2000

Sir:

Under the provisions of 35 U.S.C. § 119 and 37 C.F.R. § 1.55(a), the applicant(s) hereby claim(s) the right of priority based on the following application(s):

Country	Application No.	Filed
INDIA	666/BOM/98	October 16, 1998
EPO	98119630.6	October 16, 1998

A certified copy of the above-noted application(s) is(are) attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fee required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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GMM/MAA/csp
0147-0189P

Attachment

(Rev. 04/19/2000)

THE PATENTS ACT, 1970

IT IS HEREBY CERTIFIED THAT, the annex is a true copy of application & Complete Specification filed on 16.10.1998 in respect of Patent application No.666/BOM/98 of Frauunhofer Gesellschaft Zur Forderung Der Angewandten Forschung e.v. of Leonrodstr. 54, 80636 Munchen, Germany, German Company.

This Certified copy is issued under the Powers vested on me under section 147(1) of the Patents Act, 1970.....

..... Dated this 17.12.1999

07.01-2000

(R. V. PATEL)
DEPUTY CONTROLLER OF PATENTS & DESIGNS
MUMBAI-13.

FORM 1A

THE PATENTS ACT, 1970

APPLICATION FOR PATENT BY THE ASSIGNEE OF THE TRUE AND FIRST INVENTORS

(See Section 7)

ORIGINAL

We FRAUNHOFER GESELLSCHAFT ZUR FORDERUNG DER ANGEWANDTEN FORSCHUNG e. V. of LEONRODSTR. 54, 80636 MUNCHEN, GERMANY, GERMAN Company hereby declare : -

i) that We are in possession of an invention for MOLECULAR PATHOGENICIDE MEDIATED PLANT DISEASE RESISTANCE ;

ii) that We the said FRAUNHOFER GESELLSCHAFT ZUR FORDERUNG DER ANGEWANDTEN FORSCHUNG e. V. claim to be the assignee of FISCHER, RAINER of SENSERBACHWEG 218, 52074 AACHEN, GERMANY, GERMAN national ; SCHILLBERG, STEFAN, of BREMENBERG 24, 52072 AACHEN, GERMANY, GERMAN national ; NAHRING, JORG, of AN DER JUNKERSMUHLE 37, 52074 AACHEN, GERMANY, GERMAN national ; SACK, MARKUS of BRABANTER WEG 17, 52134 HERZOGENRATH, GERMANY, GERMAN national ; MONECKE, MICHAEL, of ROERMONDER STR. 42, 52068 AACHEN, GERMANY, GERMAN national ; LIAO, YU-CAI, of VAALSERSTR. 48, 52064 AACHEN, GERMANY, CHINESE national ; SPIEGEL, HOLGER, of BLUCHER PLATZ 52, 52062 AACHEN, GERMANY, GERMAN national ; ZIMMERMANN, SABINE, of ROCHUSSTR. 48, 52062 AACHEN, GERMANY, GERMAN national ; EMANS, NEIL of VAL DE LA BERWINNE 13, 4890 THIMISTER-CLERMONT, BE BRITISH national who claim and are believed to be the true and first inventors thereof;

Received Rs. 300/- (In Cash) Cheque/M.O./P.O. dt. 16/10/98 Vide Entry No. 5239 in the Register of Valuables, Bombay.

iii) that the complete specification filed with this application is and any amended specification which may hereafter be filed in this behalf will be true to the invention to which this application relates;

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iv) that We believe that We are entitled to a patent for the said invention having regard to the provisions of the Patents Act, 1970;

16 OCT 1998

v) that to the best of our knowledge, information and belief the facts and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

666/Bom/98
16/10/98

We request that a patent may be granted to us for the said invention.

We request that all the notices, requisitions and communications relating to this application may be sent to :-

CHANDRAKANT M. JOSHI
PATENT & TRADE MARK ATTORNEYS,
501, "VISHWANANAK" , CHAKALA ROAD,
ANDHERI (EAST), MUMBAI - 400 099.

Dated this 16th day of October, 1998.

CHANDRAKANT M. JOSHI
AGENT FOR
FRAUNHOFER GESELLSCHAFT ZUR FORDERUNG
DER ANGEWANDTEN FORSCHUNG e. V.

To
The Controller Of Patents,
The Patent Office,
Mumbai

ORIGINAL

FORM 3 A

THE PATENTS ACT, 1970
COMPLETE SPECIFICATION

(See Section 10)

MOLECULAR PATHOGENICIDE MEDIATED PLANT DISEASE RESISTANCE

FRAUNHOFER GESELLSCHAFT ZUR FORDERUNG DER ANGEWANDTEN FORSCHUNG e. V. of LEONRODSTR. 54, 80636 MUNCHEN, GERMANY, GERMAN Company

The following specification particularly describes and ascertains the nature of this invention and the manner in which it is to be performed : -

Molecular Pathogenicide Mediated Plant Disease Resistance

Field of the Invention

The present invention relates to gene constructs suitable for expressing agents to protect a plant against pathogens and the suitable proteins for such plant protection. These agents are named "molecular pathogenicides" This invention is related to the genetic engineering of plants and to means and methods for conferring pathogen resistance on a plant using a gene or genes encoding: a pathogen specific antibody and a toxic activity which blocks stages of the pathogen life cycle, pathogen replication or pathogen movement within a plant or pathogen transmission from plant to plant. The means and methods are given for the immobilisation of recombinant antibodies, antibody fusion proteins and antibody protein complexes in cellular membranes in different orientations and the display of recombinant proteins on the plant cell membrane. This invention also describes novel methods and protein binding partners for assembling protein complexes from individual polypeptide chains during expression of these proteins in vivo. Also given are the methods for activation of the molecular pathogenicides by in vivo proteolytic cleavage.

Background of the invention

Plant disease constitutes a major and ongoing threat to human food stocks and animal feed. Most crop plants are regularly exposed to one or more pathogen(s) that can cause incredible damage resulting in substantial economical losses every year. Attack by pathogens, such as viruses, bacteria, fungi, nematodes and insects and is a severe economic problem, which impacts all economically important crops, for example rice, soybean, sweet potato, wheat, potato, grape, maize and ornamental plants. Current protective measures rely heavily on chemical control measures for pathogen vectors, which have undesirable environmental consequences.

A more effective approach to protecting plants from pathogen attack is to create plants that are endogenously resistant to pathogens However, plant breeders have limited sources of resistance genes against plant diseases. This can now be

achieved using genetic engineering techniques, by providing the plant with genetic information required for affecting the pathogens and for being resistant to the disease caused by the pathogen. For example, in the case of a viral-pathogen, the host plant is resistant if it has the ability to inhibit or retard the replication of a virus, the symptoms of viral infection or the life cycle of the virus, including its transmission. "Resistant" is the opposite of "susceptible" and may be divided into three levels:

1) Full,
2) Medium,
3) Partial resistance,

A plant may be considered fully resistant when it shows no symptoms on infection and there is no evidence of pathogen replication or reproduction. The host plant may be resistant to the establishment of infection, pathogen reproduction and/or pathogen movement and transmission.

In recent years, the advances in plant 'molecular virology have enhanced the understanding of pathogen genome organisation and gene function. Moreover, genetic engineering of plants for virus resistance has recently provided new strategies for control of viral disease (Baulcombe, 1994), (Gadani et al., 1990). (Wilson, 1993). The following genes were expressed in transgenic plants in order to confer resistance: viral coat proteins, non-structural proteins of viral genomes, viral anti-sense transcripts, viral satellite RNAs, ribozymes and interferon genes (Baulcombe, 1994), (Gadani et al., 1990), (Wilson, 1993), (Harrison et al., 1987), (Namba et al., 1991), (Anderson et al., 1992). Although most of these approaches have been effective for attenuating infections, resistance was not complete and confined to a small spectrum of viral pathogens (Falk and Bruening, 1994), (Wilson, 1993) and bears significant risks (Palukaitis and Roossinck, 1996).

The major disadvantages of these methods are:

1) Host range is limited.
2) Pathogen range is limited.
3) Resistance is partial and though symptoms are delayed infection still results in the disease.
4) Resistance could be broken in case of coat protein mediated resistance and ribozyme mediated resistance
5) Expression of viral proteins can lead to enhanced pathogen activity. For example, in the case of viral coat protein mediated resistance, cross encapsidation of mild

non-pathogenic strains of virus by the expressed coat protein can occur which then leads to development of a more severe disease.

An alternative way to protect plants against pathogen infection is the generation and expression of recombinant antibodies (rAbs), which are often referred to as **"Plantibodies"**. Pathogen-specific recombinant antibodies targeted to different compartments of plant cells or different plant organs overcome many of the problems mentioned before and confer a broader spectrum of resistance to disease (Baulcombe, 1994). To achieve this, recombinant antibodies (Plückthun, 1991), (Winter and Milstein, 1991) against the target proteins have to be generated by cloning the corresponding antibody heavy and light chain genes from hybridoma cells, synthetic, semi-synthetic and immunocompetent phage display or ribosome display libraries; or by the generation of fully synthetic designer antibodies. This is followed by subsequent modification and rAb expression in different compartments of heterologous hosts such as bacteria, yeast, algae, baculovirus infected insect cells, mammalian cells and plants. For example, antibodies and antibody-fusion proteins binding to conserved functional domains of viral coat proteins, movement proteins, replicases or transmission factors can be used to inactivate such targets inside or outside the plant cell through immunomodulation. The feasibility of expressing recombinant antibodies (Plückthun, 1991), (Winter and Milstein, 1991) for the generation of resistance has been shown recently for both animal (Chen et al., 1994), (Duan et al., 1994), (Marasco et al., 1993) and plant viruses (Tavladoraki et al., 1993), (Voss et al., 1995), (Zimmermann et al., 1998). Single chain antibody fragments derived from monoclonal antibodies (scFvs) (Bird et al., 1988) directed against *Rev* (Duan et al., 1994) and gp120 (Chen et al., 1994) (Marasco et al., 1993) of HIV, inhibited HIV-replication, virion assembly and syncytia formation when expressed intracellularly, or within the ER of human cells.

Interestingly, intracellular expression of an scFv specific for the artichoke mottled crinkle virus coat protein in transgenic Tobacco caused a reduction of infection and a delay in symptom development (Tavladoraki et al., 1993). Targeting of TMV-specific full-size antibodies to the intercellular space of Tobacco plants inhibited viral infections up to 70% (Voss et al., 1995). In the latter case, plant produced antibodies showed the same specificity and affinity for TMV (Fischer et al., 1998) as the parental murine antibody. Cytosolic expression of an engineered scFv derived from this anti-

TMV antibody yielded fully resistant Tobacco plants, even under systemic infection conditions (Zimmermann et al., 1998). These studies demonstrate the potential of heterologously expressed recombinant antibodies to combat pathogens via intra- or extra-cellular modulation of pathogen proteins.

Plant cells can synthesise large amounts of antibodies that are functionally indistinguishable from the source monoclonal. For example, full-size antibodies (Düring et al., 1990), (Hiatt et al., 1989), (Voss et al., 1995), Fab-fragments (De Neve et al., 1993), scFvs (Owen et al., 1992; Zimmermann et al., 1998), (Tavladoraki et al., 1993) and dAbs (Benvenuto et al., 1991) have been successfully expressed in Tobacco, Potato (Schouten et al., 1997) or *Arabidopsis*, reaching expression levels as high as 6.8% of the total protein (Fiedler et al., 1997).

Targeting of recombinant antibodies by exploiting known protein trafficking signal sequences now permits rAb expression in the cytoplasm (scFv fragments (Tavladoraki et al., 1993; Zimmermann et al., 1998)), the endoplasmic reticulum (Fiedler et al., 1997), chloroplasts (Düring et al., 1990) and the intercellular space (Benvenuto et al., 1991; De Neve et al., 1993; Voss et al., 1995; Zimmermann et al., 1998) (full-size, Fab fragments, scFvs and single domain Abs). These results demonstrate the flexibility of the plant system to express any recombinant antibody or recombinant antibody fragments in almost all plant compartments, using targeting sequences that also may be from plants or derived from other eukaryotes.

The advantage of targeted protein expression is that the rAbs can be expressed where the pathogen is most vulnerable and where they will have the maximal protective effect. In patent application WO 96/09398 the use of antibody-fusion proteins as agents for controlling crop disease caused by pathogens is proposed. The antibody delivers a toxin which kills the pathogen in transgenic plants or when expressed or applied as an external immunotoxin. WO 96/09398 is focussed on recombinant Ab-fusion proteins – single polypeptides that are either genetically, chemically or "biochemically" linked to form an immunotoxin. However, WO 96/09398 does not provide proof of principle for antibody mediated pathogen resistance and it was doubtful whether any of the hypothetical examples in WO 96/09398 would work to the extent that a protection of plants against pathogen attack can be obtained sufficient to comply with the needs of the breeders and farmers. Thus, there is still a need of means and methods for conferring antipathogenic/predator characteristics to transgenic plants.

Summary of the Invention

The objective of this current patent application is to provide the means and methods for protecting plants, in particular monocotyledonous and dicotyledonous agricultural crops and ornamental plants, against pathogens in a more effective and environmentally sensitive manner. Accordingly, the solution to the technical problem is achieved by providing the embodiments characterised in the claims.

As will be described hereinbelow, the above-mentioned objective is met according to the invention by any one of the following or any combination of the following inventions: i) the expression of pathogen specific recombinant antibodies and parts thereof, or ii) by fusing antibodies or parts thereof to toxins, proteins, or enzymes having activity against the pathogens or to the effective parts of these toxins or enzymes, and then expressing these fusion proteins, or iii) by assembling protein complexes composed of an antibody or fragment thereof *in vivo* using the novel binding proteins described here and or iv) including a specific protease sensitive sequence, that is cleaved (e.g. in the presence of the pathogen or in a specific plant cell compartment) to release and or activate the toxic activity of any of the recombinant proteins in i) to iii), and or v) targeting or integrating any of the recombinant proteins in i) to iv) to cell membranes in any orientation. These agents are also named "molecular pathogenicides". Thus in one aspect the present invention relates to a fusion protein comprising

(a) at least one binding domain specifically recognising an epitope of a plant pathogen; and

(b) at least one further domain comprising a protein or peptide sequence which is toxic to the pathogen or detrimental to its replication, transmission or life cycle.

Said domains can be linked by covalent or non-covalent bonds. In a preferred embodiment of the fusion protein of the invention said binding domain comprises an antibody, a T-cell receptor, a pathogen specific receptor, a peptide specific for an epitope of a pathogen, or at least the binding site of any one of those.

In another aspect, the invention relates to membrane associated binding domains and further domains, respectively, as defined herein.

The fusion proteins composed of a pathogen specific antibody and toxin-molecule can be made by fusing the respective parts by genetic or biochemical means. In addition, the chimeric protein can preferably be assembled *in vivo* from its parts by the plant or expression organisms' endogenous protein machinery. In a particularly preferred and advantageous embodiment of the invention, these domains or parts thereof, fusion proteins or protein complexes can also be targeted to organelles and plant cell compartments or immobilised and membrane anchored by the addition of signal sequences and or membrane anchors. The recombinant molecular pathogenicide protein preferably contains specific protease cleavage sequences that are cleaved *in vivo*, by a plant and/or a pathogen specific protease(s), to release and or activate the toxic agent(s), or parts thereof, upon infection.

The fusion protein of the present invention can further comprise a carrier protein suitable for delivering the fusion protein or its domains into a host cell, preferably plant cell or a cellular compartment thereof. Furthermore, the fusion protein of the present invention can comprise a fluorophore such as green fluorescent protein fused to at least one of the above-described domains the fusion protein consists of. In a further aspect, the present invention relates to a pathogenicide comprising at least one binding and/or further domain as defined herein and a cellular targeting sequence and/or membrane localisation sequence and/or motif that leads to membrane anchoring. Preferably, the membrane localisation sequence is proteolytically sensitive.

Suitable membrane anchor sequences, enabling the integration of secretory recombinant antibody fusion proteins and parts thereof in the plasma membrane, include the human T cell receptor transmembrane domains (Gross and Eshhar, 1992), glyco-phosphatidyl inositol (GPI) anchors (Gerber et al., 1992), immunoglobulin superfamily membrane anchors, tetraspan family members (Tedder and Engel, 1994; Wright and Tomlinson, 1994) and any transmembrane sequence(s) from a known protein or synthesised sequences that have a similar function and can be included in the target protein by recombinant DNA technology. Fusion of a protein to these sequences would permit display of the recombinant protein on the lumenal face of organelles of the secretory or endocytic pathway or the plant cell membrane.

This has the advantage that the recombinant protein can be targeted to the intracellular space where many pathogens are most vulnerable.

In addition, the antibodies or parts thereof, or the recombinant antibody fusion proteins, or parts thereof, may be targeted to cell membranes where they could face the cytosolic side of the membrane. Suitable targeting sequences for cytoplasmic display, include the transmembrane domains of: KAR1, for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen (Kim et al., 1997), for plasma membrane integration and cytochrome b5, for ER membrane integration (Kim et al., 1997). C-terminal linkages to fatty acids using consensus amino acid sequences leading to post translational prenylation, farnesylation, palmitoylation, myristoylation or ankyrin sequence motifs can also be used. This cytoplasmic display method has the significant advantage that the recombinant proteins can be localised at the site of intracellular pathogen replication, where they will have the most potent effect. In addition, membrane localisation of proteins stabilises the protein and reduces the effect of C-terminal protein degradation *In vivo*.

Preferably, the pathogenicide of the invention comprises the fusion protein described herein.

In a particularly preferred embodiment, the present invention relates to the described pathogenicides wherein said binding domain(s) and/or said further domain(s) are capable of self assembly in vivo.

In a further embodiment, the present invention relates to a polynucleotide encoding a fusion protein or pathogenicide of the invention. Thus, the invention relates to one or more gene constructs that encode a nucleotide sequence encoding an antibody or part thereof which is specific for a pathogen and in the case of fusion proteins, for a nucleotide sequence encoding a protein, enzyme or peptide which has detrimental effects on a pathogen and ideally is toxic to the pathogen. This invention includes antibodies specific for the pathogen and or for host proteins utilised by the pathogen during its life cycle. This invention also relates to chimeric proteins that consist of an antibody, antibodies or parts thereof, which are specific for a pathogen, and a protein or peptide which has detrimental or ideally toxic effects on the pathogen and which has been constructed by biochemically linking the antibody or parts thereof to the toxin. Furthermore, the present invention relates to a vector comprising the

polynucleotide of the invention. Said vector can comprise separate polynucleotides encoding at least one of said binding domain(s) and/or said further domain(s) of the above-described fusion protein. In addition, the present invention relates to a composition comprising vectors wherein each vector contains at least one polynucleotide encoding at least one binding domain and/or at least one further domain of the fusion protein or the pathogenicide of the invention; and wherein the expression of at least two of said polynucleotides results in the production of said fusion protein or said pathogenicide or assembly of the same in vivo.

In a preferred embodiment of the vector or the composition of the invention the polynucleotide is operatively linked to regulatory sequences allowing the expression of the fusion protein, pathogenicide or the domains thereof in a host cell. Said regulatory sequence can be a constitutive, chimeric, tissue specific or inducible promoter.

Furthermore, the present invention relates to a host cell comprising any one of the above-described polynucleotides, vectors or vectors of the compositions.

In another embodiment the present invention relates to a method for the production of a molecular pathogenicide comprising:

(a) culturing the host cell of the invention under conditions suitable for the expression of the polynucleotide; and

(b) recovering the fusion protein, pathogenicide or the domains thereof from the culture.

The present invention also relates to a molecular pathogenicide obtainable by the method of the invention or encodable by the polynucleotide of the invention.

This invention also relates to *in vivo* assembled protein complexes composed of one or more discrete polypeptide chains, encoded by separate nucleotide sequences on one or more constructs, that are assembled by the plant or expression organisms protein synthesis machinery into a protein complex.

Furthermore, the present invention relates to a method for the production of pathogen resistant transgenic plants, plant cells or plant tissue comprising the introduction of a polynucleotide or vector of the invention or the vectors of the composition of the invention into the genome of a plant, plant cell or plant tissue.

The present invention also relates to a transgenic plant cell which contains stably integrated into the genome a polynucleotide or vector of the invention or the vectors of the composition of the invention or obtainable according to the method of the invention.

In addition, the present invention relates to a transgenic plant or plant tissue comprising the above-described plant cells or obtainable by the method of the invention. Encompassed are also the transgenic plants wherein the fusion protein or pathogenicide are made functional against pathogens by *in vivo* assembly after co transformation of at least two independent plant expression constructs or after sexual crossing to form hybrid offspring from two parental plants expressing one or more of the domains of the fusion protein or the pathogenicide, or any other form of genetic recombination. Preferably, the transgenic plant of the invention displays improved resistance against a pathogen that the wild type plant was susceptible to.

Furthermore, the present invention relates to harvestable parts and propagation material of a plant of the invention comprising plant cells of the invention.

In a still further embodiment, the present invention relates to a kit comprising any one of the described fusion proteins, pathogenicides, polynucleotides, compositions or molecular pathogenicides of the invention.

In another embodiment the present invention relates to the use of the described fusion proteins, polynucleotides, vectors, compositions and molecular pathogenicides of the invention in agriculture for the protection of a plant against the action of a pathogen.

Some aspects of the present invention will be described herein below.

The term "binding domain" is used to denote polypeptide chain(s) which exhibit a strong monovalent, bivalent or polyvalent binding to a given epitope or epitopes. Preferably, said binding domain is an antibody or a binding site thereof. The antibodies may be generated by hybridoma technology, or ribosome display, or phage display, of natural naïve origin, or immunised origin, semi-synthetic or fully synthetic libraries. The term "antibody" is also used to denote designer antibodies. These antibody polypeptides are encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind the given epitope or epitopes. The recognised immunoglobulin genes include the kappa and lambda light chain genes, the mu, delta, gamma, alpha and epsilon constant regions as well as all immunoglobulin variable regions from vertebrate, camelid, avian and pisces species. The term antibody, as used herein, includes in particular those antibodies synthesised or constructed *de novo* using recombinant DNA methodology, such as recombinant full-size antibodies, dimeric secretory IgA antibodies, multimeric IgM antibodies, $F(ab')_2$-fragments, Fab-fragments, Fv-fragments, single chain Fv-fragments (scFvs), bispecific scFvs, diabodies, single domain antibodies (dAb), minibodies and molecular recognition units (MRUs). Antibody sequences may be derived from any vertebrate, camelid, avian or pisces species using recombinant DNA technology, or also by using synthetic, semi-synthetic and naïve or immunocompetent phage and ribosome display libraries, gene shuffling libraries, and fully synthetic designer antibodies. In this invention, the antibodies are generated against specific pathogen or host plant epitopes that are involved in the pathogen replication, reproduction or life cycle.

The term "pathogen" is used to denote viral or virus like organisms, bacteria, mycoplasmas, fungi, insects or nematodes that affect the germination of seed, growth, development, reproduction, harvest, yield or utility of a plant.

The term "toxic" refers to an activity, which may be peptide or polypeptide encoded, that affects the reproduction or replication of a pathogen and/or any stages of its life cycle. In the case of viral pathogens, this includes entry into the plant, viral uncoating and disassembly, viral replication, viral assembly, cell to cell and long distance movement and the development, spread, or life cycle of the virus. Suitable toxic activities include RNAse (Leland et al., 1998) and DNAse, Ribosome inactivating proteins (Barbieri et al., 1993), (Girbes et al., 1996), (Hartley et al., 1998) and or toxins with antimicrobial activity (Dempsey et al., 1998) . Antibodies or recombinant

proteins in themselves are also considered toxic when they affect the pathogen by binding to pathogen and or host proteins that are utilised by a pathogen during its replication, reproduction, life cycle or transmission. For example, a fusion protein composed of a virus specific antibody and a viral coat protein will interfere with virus reproduction by both binding to the virus and by disrupting viral assembly or disassembly in the host cell.

The term "molecular pathogenicide" refers to the antibodies and proteins described in this application, which have toxic effects on pathogen(s) either as single fusion proteins, when expressed in combination with other proteins, or when expressed as part of protein complexes that are assembled *in vivo*.

Monoclonal antibodies (Köhler and Milstein, 1975) can be raised against almost any epitope or molecular structure of a pathogen or host protein using several techniques. The most common method is the hybridoma technique starting with immunocompetent B lymphocytes from the spleen or thymus which are obtained after immunisation with native antigen, recombinant antigen, antigen fusion proteins, antigen domains or by *in vitro* or genetic immunisation. In addition, recent advances in molecular biology techniques now permit the use of cloned recombinant antibody fragments and antibodies derived from mice and other organisms than the mouse. Suitable recombinant antibody fragment(s) include the complete recombinant full-size antibodies, dimeric secretory IgA antibodies, multimeric IgM antibodies, the $F(ab)_2$ fragment, the Fab-fragment, the Fv-fragment, single chain antibody fragments (scFvs), single binding domains (dAbs), a bivalent scFv (diabody) (Poljak, 1994), minibody (Carter and Merchant, 1997), bispecific scFv antibodies (Plückthun and Pack, 1997) where the antibody molecule recognises two different epitopes, (which may be from the pathogen or the host or both the pathogen and the host), triabodies and any other part of the antibody such as, molecular recognition units (MRUs), which show binding to the target epitopes. Genes encoding these suitable recombinant antibody fragment(s) may be derived from vertebrates, camelids, avian or pisces species.

Also, single chain antibodies that have affinities for pathogen or host structures and proteins can be identified using phage display libraries or ribosome display libraries, gene shuffled libraries, which can be constructed from synthetic, semi-synthetic or

naïve and immunocompetent sources (Plückthun, 1991; Winter et al., 1994; Winter and Milstein, 1991). Phage display and suitable techniques can be used to specifically identify antibodies, or fragments thereof, with the desired binding properties. Using recombinant antibody technology it is possible to identify antibodies or fragments that are highly specific for a single pathogen, or which recognise a consensus epitope conserved between several pathogens, where the antibodies will have a broad specificity against pathogens. The durability and effect of antibody mediated resistance can be improved by i) recombinant antibody affinity maturation, ii) CDR randomisation and selection, iii) stabilisation by framework optimisation of a selected pathogen specific antibody, iv) bi-specific antibody expression, v) the generation of antibody fusion proteins, or vi) the expression of antibodies in combinations with others that may potentiate their individual effects. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage displayed antibodies selections, yielding a high increment of affinity from a single library of phage antibodies which bind to an epitope of a pathogen (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J Immunol. Methods 183 (1995), 7-13).

The recombinant antibodies can be identified and utilised according to methods that are familiar to anyone of ordinary skill in the art.

Antibodies

This invention describes antibodies or fragments thereof which recognise structures of the pathogen or host plant and directly or indirectly lead to resistance or partial resistance when expressed alone or when expressed as chimeric fusion protein coupled to a toxic activity or when expressed and assembled *in vivo* with a toxic activity to form an *in vivo* assembled molecular pathogenicide protein complex.

Antibodies can be generated that recognise pathogen-specific epitopes or host plant specific epitopes which have a role in the life cycle of a pathogen. Suitable antibodies for engineering viral resistance include, but are not limited to, those binding to conserved functional domains of viral coat proteins, movement proteins, or replicases and are an approach to obtain broad-spectrum resistance and reduce the environmental risks by inactivating the targets inside and/or outside the plant cell through immunomodulation. The feasibility of this approach has been recently shown for both animal (Chen et al., 1994), (Duan et al., 1994), (Marasco et al., 1993) and

plant viral resistance (Tavladoraki et al., 1993), (Voss et al., 1995), (Zimmermann et al., 1998). These antibodies or fragments thereof may be inactivating in themselves or in combination with one or more other antibodies, or a toxin, or in combination with a carrier, transmembrane domain or signal peptide. Importantly, plant pathogen resistance can be enhanced by the co-expression of multiple antibodies.

Toxins

Toxins include all proteins and peptides that have a detrimental or toxic effect on a pathogen during its life cycle and/or an effect on the pathogen during plant infection or pathogen replication, spread or transmission. This includes toxins that specifically kill an infected host cell and so limit the spread and development of a disease.
Suitable toxins include the following:

- toxic peptide(s) which are specific for the pathogen and mediates toxicity e.g. by membrane permeabilisation based on alteration of membrane potential (Ham et al., 1994; Sangster, 1997).
- blocking peptides which bind to structural or non structural pathogen proteins, or nucleic acid motifs, and inhibit pathogen function, growth, development or toxicity to the host (Hayakawa, 1991; Silburn et al., 1998).
- peptide mimics that bind to pathogen or host protein motifs and that modulate or block the pathogen's replication, e.g. peptide derivatives of proteinase inhibitors that play a physiological role as inhibitors of viral replication and can be used as antiviral agents (Bjorck et al., 1990), (Bjorck et al., 1989).
- binding domains, such as antibodies defined above specifically recognising an epitope of a plant pathogen.
- peptide mimics that bind to pathogen or host protein motifs and that modulate or block the pathogen's movement within the host plant. As an example, the BC peptide, which mimics the nuclear localisation signal region of HIV-1, reduces HIV-1 production by 75% when expressed in infected dividing cultured human T-cells (Friedler et al., 1998).
- toxins which kill the host cell where the pathogen is replicating and has penetrated the cytosol (Barbieri et al., 1993; Hartley et al., 1996; Madshus and Stenmark, 1992), for example (Ribosome Inactivating proteins) RIPs

which enter the cytosol and are among the most potent cytotoxins known. Ribosome-inactivation is achieved in all cases through the cleavage of an N-glycosidic bond between ribose and a specific adenine residue in the universally conserved sequence 5'-AGUACGA*GAGGA-3' (where A* indicates the target adenine) located 250-400nt from the 3' end of 23S/25S/28S rRNAs (Endo and Tsurugi, 1987), (Hartley et al., 1996). Ribosomes depurinated in this manner are unable to bind the EF-2/GTP complex and protein synthesis is blocked at the translocation step (Montanaro et al., 1975). A single RIP molecule is able to depurinate 1000-2000 mammalian cell ribosomes per min under physiological conditions (Eiklid et al., 1980; Endo and Tsurugi, 1988).

- proteins and enzymes such as RNase A that are potent cytotoxins (Leland et al., 1998). These cytotoxic ribonucleases degrade cellular RNA and cause cell death and can be used to kill infected cells and so prevent the proliferation and spread of a pathogen.

These are examples of proteins which will inhibit the replication of a pathogen at a RNA, DNA or protein level by either binding directly to a pathogen protein, replication intermediate or a host factor that is necessary for pathogen replication or movement or transmission and the pathogen life cycle. This strategy is particularly suitable for inactivating viral pathogens. In addition, we describe toxins, such as RIPs or RNase A, that are suitable for causing cell death on pathogen entry and so halting the spread of infection or proliferation of a pathogen.

In principle all antibodies, proteins, peptides and enzymes that have an activity, that may or may not be enzymatic, which are able to interfere with pathogen life cycles are suitable as part of the present constructs.

In a preferred embodiment of the present invention said enzyme is chitinase or glucanase, glucose oxidase, superoxide dismutase, DNAse or RNAse or RIP or active fragments thereof either singly or in any combination(s).

Constructs

Gene constructs may comprise the following or any combination of the follow and may be encoded on one or more plasmids: Gene constructs may comprise a nucleotide sequence or nucleotide sequences encoding complete recombinant full-

size antibodies, dimeric secretory IgA antibodies, multimeric IgM_2 antibodies, the $F(ab')_2$ fragment, the Fab-fragment, the Fv-fragment, single chain antibody fragments (scFvs), single binding domains (dAbs), a bivalent scFv (diabody) (Poljak, 1994), minibody (Carter and Merchant, 1997), bispecific scFv antibodies (Plückthun and Pack, 1997) where the antibody molecule recognises two different epitopes that may come from the pathogen or the host or both, trIabodies and any other part of the antibody (molecular recognition units (MRUs)) which shows binding to the target epitopes. Genes encoding these suitable recombinant antibody fragment(s) may be derived from vertebrates, camelids, avian or piscos species.

In the constructs according to the invention, the antibody is fused to a complete sequence of a toxic agent or a part thereof which still has activity, or which is still functionally active. Also, the chimeric protein may be encoded by nucleotide sequences on one or more constructs and may be assembled *in vivo* by the plant or expression organisms protein assembly and translation machinery. The chimeric protein can also be obtained by biochemical assembly or *In vitro* or *in vivo* assembly of the chimeric immunotoxin subunits using the cells endogenous protein assembly machinery.

The antibody, antibodies or fragments thereof are fused directly to the toxic agent or linked by a flexible spacer which does not interfere with the structure or function of the two proteins. Such flexible linkers include copies of the (Glycine-Glycine- Glycine-Glycine-Serine)$_n$ linker, where n is 1 to 4 or more copies of the linker unit, the Genex 212 and 218 linker and the flexible linker peptide of *Trichoderma reesi* cellobiohydrolase I (CBHI) (Turner et al., 1997), (Tang et al., 1996).

Constructs for cellular targeting and membrane localisation

In this invention, this targeting approach has the advantage that the molecular pathogenicide or antibody or fragment thereof can be expressed where the pathogen is most vulnerable to the action of the molecular pathogenicide and/or antibody or fragment thereof.

The desired cellular location of the molecular pathogenicide, or any components thereof, can be achieved by using the appropriate cellular targeting signals, these include but are not limited to signal peptides, targeting sequences, retention signals, membrane anchors, post translational modifications and/or membrane

transmembrane domains that target the protein to the desired organelle, desired membrane (plasma membrane, ER, Golgi, nucleus, chloroplast or vacuole) or desired membrane orientation (cytoplasmic or lumenal or plant cell membrane display) (Kim et al., 1997; Rose and Fink, 1987). Localisation sequences can be targeting sequences which are described, for example in chapter 35 (protein targeting) of L. Stryer *Biochemistry* 4th edition, W.H. Freeman, 1995. Proteins synthesised without a functional signal peptide are not co-translationally inserted into the secretory pathway and remain in the cytosol. Proteins that carry a signal peptide that directs them to the secretory pathway, which may include a transmembrane sequence or membrane anchor, will be targeted for secretion by default or reside in their target membrane organelles. Targeting signals can direct proteins to the ER, retain them in the ER (LYSLYS motif and KDEL), TGN 38, or will target proteins to cell organelles such as the chloroplasts, vacuole, nucleus, nuclear membrane, peroxisomes and mitochondria. Examples for signal sequences and targeting peptides are described in (von Heijne, 1985) (Bennett and Osteryoung, 1991) (Florack et al., 1994). In addition, the targeting signals may be cryptic and encoded by a host plant cell or heterologous eukaryotic cell proteins or animal proteins where the localisation is known and where the protein can be cloned. By constructing a fusion protein with this protein, a molecular pathogenicide can be targeted to the localisation of the protein without the need for identification of the cryptic targeting signal. Suitable cryptic signals are those encoded by the resident Golgi enzymes.

The molecular pathogenicides described in this invention can be targeted to cellular membranes by incorporating heterologous sequences into the recombinant protein which permit its synthesis as a membrane protein or as a membrane associated protein or its post translational modification to associate it with cellular membranes. Suitable membrane anchor sequences, enabling the integration of recombinant antibody fusion proteins and parts thereof in the plasma membrane, include the human T cell receptor transmembrane domains (Gross and Eshhar, 1992), glyco phosphatidyl inositol (GPI) anchors (Gerber et al., 1992), Immunoglobulin superfamily membrane anchors, tetraspan family members (Tedder and Engel, 1994; Wright and Tomlinson, 1994) and any transmembrane sequence(s) from a known protein or synthesised sequences that have a similar function and can be included in the target protein by recombinant DNA technology.

In addition, the antibodies or parts thereof, or the recombinant antibody fusion proteins, or parts thereof, may be targeted to cell membranes where they could face the cytosolic side of the membrane. Suitable targeting sequences for cytoplasmic display, include the transmembrane domains of: KAR1, for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen (Kim et al., 1997), for plasma membrane integration and cytochrome b5, for ER membrane integration (Kim et al., 1997). C-terminal linkages to fatty acids using consensus amino acid sequences leading to post translational prenylation, farnesylation, palmitoylation, myristoylation or ankyrin sequence motifs can also be used.

Constructs for antibody stabilisation by membrane display

Pathogen-specific recombinant antibodies can be fused to different transmembrane anchors to improve the expression levels and stability of these molecules inside the plant cell, by targeting the expressed recombinant protein to cell membranes in various orientations. This can be accomplished by adding:

a) C-terminal localisation sequences to target and integrate recombinant cytosolic proteins with N-terminal leader peptides into the bilayer of cellular membranes, thus facing to the plant apoplast. Suitable membrane localisation sequences include the human T cell receptor β chain transmembrane domain and the human platelet derived growth factor receptor (PDGFR) transmembrane domain, glycophosphatidyl inositol (GPI) anchors, immunoglobulin superfamily membrane anchors and any transmembrane sequence(s) from a known protein or synthesised sequences that have a similar function and can be included in the target protein by recombinant DNA technology.

b) Amino terminal transmembrane proteins with either dual or tetrameric plasma membrane spanning domains to expose both the N- and C-termini of secretory recombinant proteins to the cytosol. This can be achieved by using suitable members of the tetraspan family including CD9, CD20, CD81 and the In-Hc-Ic dualspan typeII-IV hybrid of the MHC invariant chain and H-2^{o} hybrid protein. This method enables the orientation of a secreted and membrane anchored antibody construct with its N- and C-terminus into the cytosol. Alternatively fusions to SNAP-25 can be used for the same orientation.

c) C-terminal anchor sequences to target and integrate recombinant cytosolic proteins without N-terminal leader peptides into the bilayer of endomembranes posttranslationally. Suitable targeting sequences include transmembrane domains of KAR1 for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen for plasma membrane integration (Kim et al., 1997) and cytochrome b5 for ER membrane integration (Kim et al., 1997).

d) Addition of consensus motifs to the protein that permit C-terminal linkages to fatty acids by prenylation, farnesylation, palmitoylation, myristoylation in the cytosol which then lead to membrane integration.

e) Addition of ankyrin sequence motifs (Lambert and Bennett, 1993; Peters and Lux, 1993).

Constructs for *in vivo* protein complex assembly

In addition, the antibody or fragment thereof can be encoded by a separate nucleotide sequence to that of the toxin and the antibody and toxin, either of which may encode membrane localisation or cellular targeting sequences, can be encoded by one or more vectors, e.g., plasmids. The constructs contain nucleotide sequences encoding complimentary binding proteins so that when the antibody, or fragment thereof, is genetically fused to one binding partner and the toxin, or fragment thereof, is genetically fused to the second binding partner, these two independent proteins will bear mutually recognising binding activities. When these two independent proteins are expressed in the same plant compartment, the binding domains will bind to form a molecular pathogenicide with two subunits and similar properties to an antibody-toxin fusion protein. Suitable binding domains/partners include:

- A single chain antibody and its corresponding epitope, where the single chain binds to the epitope and thereby enables binding between two independent proteins,
- leucine zippers (Carter et al., 1995),
- Antibody heavy and light chains, where one protein is fused to the heavy chain and assembly of heavy and light chain takes place in the ER,
- other homo- or hetero-binding domains.

Anyone of ordinary skill in the art will recognise that the component antibody, antibodies or fragments thereof or component pathogen binding peptides, as described, and component toxin or fragments thereof can each bear a binding partner. When expressed in the same compartment of a plant, or when encountering each other, these binding domains can then permit the assembly of a molecular pathogenicide with all the properties required from the components. Anyone skilled in the art will recognise that this can be achieved by other means than those described above which are intended as examples to better illustrate the principle of *in vivo* assembly and are not intended to be taken as a limiting or a comprehensive description.

Carrier proteins

Anyone skilled in the art will also recognise that the various components of the present invention can be expressed in such a way that they are on the surface of a third carrier protein, suitable carriers include glutathione S-transferase (GST) encoded by *Schistosoma japonicum* (Smith and Johnson, 1988), TMV coat protein, maltose-binding protein and thioredoxin (LaVallie et al., 1993) or other proteins.

In addition, any of the components of the present invention may be tagged with a genetically encoded fluorophore, suitable fluorophores include, but are not limited to, the green fluorescent protein (GFP) from *Aequoria victoria*. This approach would be especially useful for monitoring the localisation of a pathogen or molecular pathogenicide during infection.

If the fusion protein or proteins are expressed in a heterologous organism for production of the protein or proteins, it may be necessary to modify the gene construct in order to match the codon preference of the organism and to remove mRNA motifs that reduce the stability of the transcript.

All of the components of the molecular pathogenicides described in this invention can be separately transformed into plant lines which can then be sexually crossed to give offspring that product the molecular pathogenicides in a functional form.

Anyone skilled in the art will recognise that the antibodies, peptides and toxins can be combined in several forms and encoded on different plasmids to produce proteins that have the desired effect on the pathogen. Anyone skilled in the art will also

recognise that assembling the molecular pathogenicides from individually genetically encoded subunits can be achieved by several methods.

Target pathogens

Viruses, bacteria, mycoplasmas, fungi, nematodes, insects and other pathogens.

Vectors

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain a polynucleotide according to the invention or any one of the above-described gene constructs. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the polynucleotides and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

In a preferred embodiment the polynucleotide present in the vector is linked to regulatory elements which allow the expression of the polynucleotide in prokaryotic and/or eukaryotic cells. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotic and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise normally promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilisation of the transcript, for example, those of the 35S RNA from Cauliflower Mosaic Virus (CaMV). In this respect, the person skilled in the art will readily appreciate that the polynucleotides encoding at least one of the above-described domains of the fusion proteins or pathogenicide of the invention may encode all of the domains or only one. Likewise, said polynucleotides may be under the control of the same promoter or may be separately controlled for expression. Other promoters commonly used are the Figwort Mosaic virus promoter, the

polyubiquitin promoter, and the actin promoter for ubiquitous expression. The termination signals usually employed are from the Nopaline Synthase promoter or from the CaMV 35S promoter. A plant translational enhancer often used is the TMV omega sequences, the inclusion of an intron (Intron-1 from the Shrunken gene of maize, for example) has been shown to increase expression levels by up to 100-fold. (Maiti et al., Transgenic Research 6 (1997), 143-156; Ni et al., Plant Journal 7 (1995), 661-676). Additional regulatory elements may include transcriptional as well as translational enhancers. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the P_L, *lac*, *trp* or *tac* promoter in *E. coli*, and examples for regulatory elements permitting expression in eukaryotic host cells are the *AOX1* or *GAL1* promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1 (GIBCO BRL). Advantageously, the above-described vectors of the invention comprises a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed hosts, for example plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hygro, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilise indole in place of tryptophan; hisD, which allows cells to utilise histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilise mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).

Useful scorable marker are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a vector of the invention.

Host cells and expression of fusion proteins and pathogenicides

The present invention furthermore relates to host cells comprising a vector as described above or a polynucleotide according to the invention. The vector or polynucleotide according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extrachromosomally.

The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred fungal cells are, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae.

Another subject of the invention is a method for the preparation of the above-described fusion proteins and pathogenicides which comprises the cultivation of host cells according to the invention which, due to the presence of a vector or a polynucleotide according to the invention, are able to express such a protein, under conditions which allow expression and optionally assembly of the fusion protein or pathogenicide and recovering of the so-produced protein from the culture. Depending on the specific constructs and conditions used, the protein may be recovered from the cells, from the culture medium or from both. For the person skilled in the art it is well known that it is not only possible to express a native protein but also to express the protein as fusion polypeptides or to add signal sequences directing the protein to specific compartments of the host cell, e.g., ensuring secretion of the peptide into the culture medium, etc. Furthermore, such a protein and fragments thereof can be chemically synthesised and/or modified according to standard methods described, for example herein.

The present invention furthermore relates to molecular pathogenicides encoded by the polynucleotides according to the invention or produced by the above-described method. In this context, it is also understood that the fusion proteins and pathogenicides according to the invention may be further modified by conventional methods known in the art.

Plant promoters and expression control elements

The fusion constructs are expressed in plants either stably in transgenic plants or transiently under the control of any type of promoter that is active in plants. For long-term resistance in host plants, high yield production of recombinant proteins, stable expression is preferred.

In general, such regulatory elements comprise a promoter active in plant cells. To obtain expression in all tissues of a transgenic plant, preferably constitutive promoters are used, such as the 35 S promoter of CaMV (Odell, Nature 313 (1985), 810-812) or promoters of the polyubiquitin genes of maize (Christensen, Plant Mol. Biol. 18 (1992), 675-689). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus, EMBO J. 8 (1989), 2245-2251). Further examples are:

a) Expression control elements (*e.g.* promoters listed below in b to f, enhancer sequences, transcriptional and translational enhancers, transcription terminators, polyadenylation sites etc.) and a selectable marker if necessary.
b) Constitutive promoters such as the CaMV-35S (Benfey et al., 1989) and the *nos* promoter (Mitra and Gynheung, 1989).
c) Viral subgenomic promoters.
d) Tissue specific promoters and chimeric promoters (Ni et al., 1995), (Comai et al., 1990).
e) Inducible promoters (Caddick et al., 1998).
f) Transient expression systems (Kapila et al., 1997).

Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, Vicia, wheat, barley etc. Inducible promoters may be used in order to be able to exactly control expression. An example for inducible promoters are the promoters of genes encoding heat shock proteins. Also microspore-specific regulatory elements and their uses have been described (WO96/16182).

Furthermore, the chemically inducible Tet-system may be employed (Gatz, Mol. Gen. Genet. 227 (1991); 229-237). Further suitable promoters are known to the person skilled in the art and are described, e.g., in Ward (Plant Mol. Biol. 22 (1993), 361-366). The regulatory elements may further comprise transcriptional and/or translational enhancers functional in plants cells. Furthermore, the regulatory elements may include transcription termination signals, such as a poly-A signal, which lead to the addition of a poly A tail to tho transcript which may improve its stability.

Furthermore, it is in principle possible to modify the coding sequence in such a way that the protein is located in any desired compartment of the plant cell. These include the endoplasmatic reticulum, the vacuole, the mitochondria, the plastids, the apoplast, the cytoplasm etc. Methods how to carry out this modifications and signal sequences ensuring localisation in a desired compartment are well known to the person skilled in the art.

Transformation

Methods for the introduction of foreign DNA into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow for stably integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilise enzymes capable of promoting homologous recombination in plants (see, e.g. WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors

are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).
Suitable strains of Agrobacterium tumefaciens and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialised vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of Agrobacterium tumefaciens is preferred in the method of the invention, other Agrobacterium strains, such as Agrobacterium rhizogenes, may be used, for example if a phenotype conferred by said strain is desired.
Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou (1996) Trends in Plant Science 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995).
The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc.

Transformation can be done using any method that leads to expression of construct or constructs in a plant and these methods can be used for stable transformation where the gene of interest is incorporated in the host plant DNA or where the construct is transiently expressed. Examples of transformation technology include:

a) *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* mediated transformation (Turpen et al., 1993; White, 1992): based on the insertion of a

foreign DNA sequence into the plant genome carried on a plasmid DNA within the agrobacteria. The foreign gene is inserted into the plant genome together with bacterial plasmid sequences.

b) Particle bombardment (Sanford et al., 1990), (Klein and Fitzpatrick-McElligott, 1993) or biolistic process (Furth, 1997): Particle bombardment uses particles coated with the DNA that penetrate the plant cell at high velocity and the DNA is incorporated into the host genome by host recombination processes. Besides particle bombardment biollstic processes also include injection methods.

c) Tissue electroporation (Chowrira et al., 1995; D'Halluin et al., 1992): under the influence of an electric field, DNA enters pores in the plant cell membrane and is incorporated into the plant genome by recombination.

d) Use of liposomes or methods which increase the uptake of free DNA (Spörlein and Koop, 1991; White, 1992).

e) Any methods for integration of foreign DNA in a plant cell resulting in transiently or stably transformed plants.

Target plants

The present invention relates to transgenic plant cells which contain a polynucleotide, vector or composition of vectors of the invention. Preferably, said polynucleotide or vector is stably integrated into the genome.

As is immediately evident to the person skilled in the art, the vectors of the present invention can carry nucleic acid molecules encoding the domains of the fusion protein or pathogenicide of the invention either alone or in combination. The same applies to the above described plant cells, plant tissue and plants transformed therewith. Likewise, said nucleic acid molecules may be under the control of the same regulatory elements or may be separately controlled for expression. In this respect, the person skilled in the art will readily appreciate that the nucleic acid molecules encoding the domains of the fusion protein or pathogenicide can be expressed in the form of a single mRNA as transcriptional and optionally translational fusions. This means that domains are produced as separate polypeptides or in the latter option as a fusion polypeptide that is further processed into the individual proteins, for example via a cleavage site for proteinases that has been incorporated between the amino acid sequences of both proteins. The resultant protein domains can then self-assemble in vivo. Of course, the

domains may also be expressed as a bi- or multifunctional polypeptide, preferably disposed by a peptide linker which advantageously allows for sufficient flexibility of both proteins. Preferably said peptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of one of said proteins and the N-terminal end of the other of said proteins when said polypeptide assumes a conformation suitable for biological activity of both proteins when disposed in aqueous solution in the plant cell. Examples of the above-described expression strategies can be found in the literature, e.g., for dicistronic mRNA (Reinitiation) in Hefferon, J. Gen. Virol. 78 (1997), 3051-3059, fusion proteins are described in Brinck-Peterson, Plant Mol. Biol. 32 (1996), 611-620 and Hotze, FEBS Lett. 374 (1995), 345-350; bifunctional proteins are discussed in Lamp, Biochem. Biophys. Res. Com. 244 (1998), 110-114 and Dumas, FEBS Lett. 408 (1997), 156-160 and for linker peptide and protease it is referred to Doskeland, Biochem. J. 313 (1996), 409-414.

In a preferred embodiment of the invention, the transgenic plant cell comprises a selectable marker. As described above, various selectable markers can be employed in accordance with the present invention. Advantageously, selectable markers may be used that are suitable for direct selection of transformed plants, for example, the phophinothricin-N-acetyltransferase gene the gene product of which detoxifies the herbicide L-phosphinothricin (glufosinate or BASTA); see, e.g., De Block, EMBO J 6 (1987), 2513-2518 and Dröge, Planta 187 (1992), 142-151.

The presence and expression of the polynucleotides or vectors in the transgenic plant cells leads to the synthesis of a fusion protein or pathogenicide of the invention or assembly of the same which has an influence on pathogen resistance in plants containing such cells.

Thus, the present invention also relates to transgenic plants and plant tissue comprising transgenic plant cells according to the invention. Due to the expression of a fusion protein or pathogenicide of the invention or their domains, e.g., in cellular compartments and/or plant tissue these transgenic plants may show various physiological, developmental and/or morphological modifications in comparison to wild-type plants.

Advantageously, these transgenic plants display a resistance against a pathogen that the corresponding wild type plant was susceptible to.

In general, the plants which can be modified according to the invention can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, pea nut, soy bean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

In yet another aspect, the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention. Harvestable parts can be in principle any useful parts of a plant, for example, leaves, stems, fruit, flowers, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

Kits

In addition, the present invention relates to a kit comprising the above-described fusion protein, pathogenicide, polynucleotide or vectors. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic plant cells, plant tissue or plants. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art. The kit or its ingredients according to the invention can be used in plant cell and plant tissue culture, for example in agriculture. The kit of the invention and its ingredients are expected to be very useful in breeding new varieties of, for example, plants which display improved properties such as those described herein.

It is also immediately evident to the person skilled in the art that the polynucleotides and vectors of the present invention can be employed to produce transgenic plants with a further desired trait due to genetic engineering (see for review TIPTEC, Plant Product & Crop Biotechnology 13 (1995), 312-397). This can be, for example, an acquired resistance to other pathogens or quality improvements of the plants comprising (i) herbicide tolerance (DE-A-3701623; Stalker, Science 242 (1988), 419), (ii) insect resistance (Vaek, Plant Cell 5 (1987), 159-169), (iii) virus resistance (Powell, Science 232 (1986), 738-743; Pappu, World Journal of Microbiology & Biotechnology 11 (1995), 426-437; Lawson, Phytopathology 86 (1996), 56 suppl.), (vi) ozone resistance (Van Camp, BioTech. 12 (1994), 165-168), (v) improving the preserving of fruits (Oeller, Science 254 (1991), 437-439), (vi) improvement of starch composition and/or production (Stark, Science 242 (1992), 419; Visser, Mol. Gen Genet. 225 (1991), 289-296), (vii) altering lipid composition (Voelker, Science 257 (1992), 72-74), (viii) production of (bio)polymers (Poirer, Science 256 (1992), 520-523), (ix) alteration of the flower colour, e g by manipulating the anthocyanin and flavonoid biosynthetic pathway (Meyer, Nature 330 (1987), 667-678, WO90/12084), (x) resistance to bacteria, insects and fungi (Duering, Molecular Breeding 2 (1996), 297-305; Strittmatter, Bio/Technology 13 (1995), 1085-1089; Estruch, Nature Biotechnology 15 (1997), 137-141), (xi) inducing and maintaining male and/or female sterility (EP-A1 0 412 006; EP-A1 0 223 399; WO93/25695) and (xii) remediation of contaminated soils (Cunningham, TIBTECH 13 (1995), 393-397).

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilised which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for

retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

Description of the figures

Figure 1 shows a description of various orientations for molecular pathogenicide display on cellular membranes. Recombinant molecular pathogenicides can be targeted by cellular signals and expressed in several orientations on cellular membranes, for example: A: where the recombinant protein faces the cytospl or extracellular space after fusion to a transmembrane domain or after post translational lipid modification and B: where the recombinant protein is fused to a protein with 4 transmembrane domains. In C and D possible orientations of toxins are displayed. In addition, the toxin and or recombinant antibody fragment can be fused to the c terminal of any of the example protein structures.
N: protein amino terminal; C: protein carboxy terminal; tm: transmembrane domain; rAb: recombinant antibody fragment or binding domain.

Figure 2 shows example constructs for membrane anchoring of scFv24 in the plant cell plasma membrane (see example 1). 35SS: 35S promoter from Cauliflower Mosaic Virus with duplicated enhancer; CHS 5'-UT: chalcone synthase 5' untranslated region; Leader peptide: original murine leader sequence from the parental monoclonal antibody 24 light chain; V_L: Variable domain of the parental monoclonal antibody 24 light chain; V_H: V_L: Variable domain of the parental monoclonal antibody 24 heavy chain; Linker: 14 amino acid linker sequence; c-myc. c-myc epitope tag sequence; TcRβ: Human T cell receptor β chain; PDGFRTM: Platelet derived growth factor receptor transmembrane domain; Term: termination sequence from Cauliflower mosaic virus.

Figure 3 shows example constructs for molecular pathogenicide display facing the cell cytoplasm. 35SS: 35S promoter from Cauliflower Mosaic Virus with duplicated enhancer; CHS 5'-UT: chalcone synthase 5' untranslated region; VL: Variable domain of the parental monoclonal antibody 24 light chain; VH: Variable domain of the parental monoclonal antibody 24 heavy chain; Linker 1: 14 amino acid linker

(Genex 212) sequence; Linker 2: 10 amino acid linker (Gly4Ser)2 sequence; Term: termination sequence from Cauliflower mosaic virus.

Figure 4 shows example constructs for viral coat protein antibody fusion proteins and various potential carrier antibody-protein fusion proteins. scFv24: single chain antibody derived from parental monoclonal mAb24 recognising a neotope on the surface of intact TMV virions; GST: Glutathione S-transferase from *Schistosoma Japonicum*; Omega sequence: Tobacco Mosaic virus 5' untranslated region; linker: 10 Amino acid $(Gly_4Ser)_2$ linker sequence; His6: 6 histidine residue epitope tag sequence; 35SS: 35S promoter from Cauliflower Mosaic Virus with duplicated enhancer; TRXec: Thioredoxin from *Escherichia coli*; TRXnt: Thioredoxin from *Nicotiana tabacum*; CP: coat protein monomer from Tobacco mosaic virus; TMV 3' UT: Tobacco Mosaic virus 3' untranslated region.

Figure 5 shows the strategy and example constructs for *in vivo* molecular pathogenicide assembly using an antibody: antigen interaction as the binding partners for *in vivo* assembly. The two binding partners are an epitope tag and a high affinity antibody which specifically recognises this epitope tag. To assemble a molecular pathogenicide protein complex, the epitope specific antibody is genetically fused to a pathogen specific antibody and the epitope tag is genetically fused to the toxin sequence. Both of these recombinant proteins are then expressed in the same cell compartment. The epitope specific antibody binds the epitope expressed on the surface of the toxin. This high affinity interaction then gives a molecular pathogenicide protein complex, which specifically recognises the pathogen and bears a toxic activity. Linker 4 can encode specific protease cleavage sites.
The epitope and pathogen specific antibodies can also be included in the constructs in the same orientation but where the epitope specific antibody precedes the pathogen specific antibody in the 5' to 3' direction.

A: schematic of molecular pathogenicide protein complex assembly in a cell compartment; B: Example constructs showing two possible arrangements (Ab1 and Ab2) of the individual V_L and V_H domains of both the pathogen specific and epitope specific antibody fragments; C: two possible arrangements (Tox 1 and Tox2) for epitope toxin fusion proteins.

Figure 6 shows the strategy and example constructs for *in vivo* molecular pathogenicide assembly using an antibody heavy chain: antibody light chain interaction as the binding partners for *In vivo* assembly. The two binding partners are an antibody heavy chain and an antibody light chain which specifically recognises this epitope tag. To assemble a molecular pathogenicide protein complex, the epitope specific antibody is genetically fused to a pathogen specific antibody heavy chain c-terminus. Both of these recombinant antibody heavy chain and light chains are then expressed in the same cell compartment, where they assemble via disulphide bridges to give a molecular pathogenicide protein complex, which specifically recognises the pathogen and bears a toxic activity. Linker 1 can encode specific protease cleavage sites. Also, the toxin can fused to the N-terminus of the antibody heavy chain using linker 1, or the N or C terminus of the light chain.

A: schematic of the final assembled molecular pathogenicide. B: example constructs.

Examples

The following examples are given to better describe the practice and applications of the present invention and should not be considered to be a limiting description nor interpreted to limit the scope and applications of the present invention. Those skilled in the art will recognise that various modifications can be made to the methods and genes described here without substantively departing from the spirit and scope of the present invention.

Example 1

Expression of a membrane integrated anti-viral antibody

Plasma membrane targeted expression of a recombinant antibody against the coat protein of Tobacco Mosaic virus (TMV)

The following steps are taken:

1) Antibodies against the coat protein of TMV, intact virions or specific coat protein peptides and monoclonals are generated by hybridoma technology.

2) Hybridoma cell lines are cloned and cDNA sequences encoding the antibody heavy and light chains are cloned to generate a recombinant antibody or any recombinant version thereof. This is achieved using antibody heavy and light specific oligonucleotides and the reverse transcriptase polymerase chain reaction using isolated mRNA from a single hybridoma clone. This permits cloning of the full size antibody.

3) The cloned full size specific antibody heavy and light chain cDNAs from step 2 are used as a template for amplification of the heavy and light chain variable domains using specific oligonucleotide primers including a linker peptide sequence (i.e. GENEX 212) and splice overlap extension polymerase chain reaction. This step then provides the single chain antibody fragment and the two variable domains are linked by a 14 amino acid sequence.

4) The recombinant scFv gene from step 3 is inserted in a microbial or eukaryotic expression vector.

5) The binding specificity and function of the recombinant scFv (i.e. specificity and affinity for the target antigen) is checked after expression of the construct from step 4 in a heterologous host, such as in the periplasm of *E. coli*, using ELISA, surface plasmon resonance or western blotting.

6) A signal sequence is added to the 5' end of the recombinant scFv nucleotide sequence from step 3. A 3' linker peptide sequence (human T cell receptor constant domain) is added and this is then followed by the addition of a 3' transmembrane sequence from the human T cell receptor chain. Suitable membrane localisation sequences also include the platelet derived growth factor receptor (PDGFR) transmembrane domain.

7) The 5' untranslated region from chalcone synthase is added to the 5' end of the construct from step 6.

8) The chimeric gene from step 7 is then inserted into a plant expression vector, such as pSS (Voss et al., 1995), upstream of the 3' untranslated region from Cauliflower mosaic virus, or any other source and the termination region from Cauliflower mosaic virus downstream of the 35SS promoter (Fig.2). This vector also contains a selectable marker. In case of markerless and vectorless gene transfer selection marker sequences can be omitted.

9) *Agrobacterium tumefaciens* is transformed by N_2 transformation with the construct from step 8.

10) Expression and function of the recombinant scFv construct in plants are checked by transient expression in plant cells and ELISA, surface plasmon resonance or western blotting.

11) Transgenic plants are generated by transferring the construct from step 8, and a screenable selection marker, which is present in the pSS expression vector (e.g. the NPT-II gene for kanamycin resistance), into the plant genome by Agrobacteria mediated transformation.

12) Regenerated plants are screened using the selection marker for integration of the fusion gene.

13) Expression of the fusion protein in regenerated plants is followed by western blotting cell extracts, ELISA or surface plasmon resonance analysis.

14) The activity of the expressed fusion protein (i.e. affinity and specificity) is checked by ELISA using intact TMV virions as the antigen.

15) Localisation of the fusion protein is checked by indirect immuno-fluorescence, or confocal microscopy or immuno-electron microscopy.

16) The activity of the antibody in generating resistance against viruses is assayed by viral infection bioassays on transgenic plants, generated in steps 11 to 12 by using virions or infectious transcripts.

The orientation of Type II or tetraspan membrane protein can be exploited to permit display of molecular pathogenicdes to the cytoplasm after their synthesis in the secretory pathway. For cytoplasmic display of the recombinant scFv, steps 6) to 16) of example 1 are repeated with the following adaptations. The C-terminal membrane localisation sequence including the linker sequence and leader sequence of step 6 in example 1 are removed and a suitable linker and N-terminal targeting sequence belonging to the tetraspan family is added to the pathogen specific recombinant antibody to target and posttranslationally integrate recombinant proteins into the bilayer of plasma membranes. Suitable members of the tetraspan family include CD9, CD20, CD81 and the In-Hc-Ic dualspan typeII-IV hybrid of the MHC invariant chain and H-2^{o} hybrid protein. This method enables the orientation of a secreted and membrane anchored antibody construct with its N- and C-terminus into the cytosol.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised and cloned against structural and

non structural proteins of any pathogen. Membrane anchor sequence(s) can be substituted against any sequence that facilitates membranes integration and provides a biological function. Moreover, example 1 can be combined with expression of examples 2-8 in any combination(s) to give high level resistance to disease.

Example 2

Expression of a neutralising anti-viral antibody with a C-terminal membrane localisation sequence

Cytoplasmic presentation of a membrane localised recombinant antibody against the coat protein of Tobacco Mosaic virus (TMV)

The steps 1) to 2) of example 1 are repeated with the following adaptations.

1) The N-terminal signal sequence is removed and replaced by a start codon.
2) The C-terminal membrane localisation sequence including the linker sequence of example 1 are replaced by suitable linker and C-terminal targeting sequences to posttranslationally target and integrate recombinant proteins into the bilayer of endomembranes. Suitable targeting sequences include transmembrane domains of KAR1 for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen for plasma membrane integration (Kim et al., 1997) and cytochrome b5 for ER membrane integration (Kim et al., 1997). Moreover, prenylation, farnesylation, palmitoylation, myristoylation and ankyrin sequence motifs can be incorporated.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised against structural and non structural proteins of any pathogen. Membrane anchor sequence(s) can be substituted against any sequence that facilitates membranes integration and provides a biological function. Moreover, example 2 can be combined with examples 1 and 3-8 in any combination(s).

Example 3

Viral resistance by expression of a molecular pathogenicide

Fusion of a nuclease activity to a recombinant antibody specific for TMV

The steps 1) to 16) of example 1 and/or the steps 1) to 2) of example 2 are repeated with the following adaptations.

1) The plant expression construct contains a 5' signal sequence to enable delivery of the recombinant to the ER lumen and then secretion to the apoplast.
2) The transmembrane targeting domain is replaced by linker coupling the protein to a C-terminal fusion with a toxin – in this case an RNAse enzyme which degrades cellular RNA, viral RNA or replicative forms and/or replicative intermediates.
3) Upon binding to the virions in the apoplast, the fusion protein will enter the cytosol of damaged cells, where the cytotoxic RNase will degrade viral RNA, replicative intermediates and replicative forms or/and cellular RNA and cause cell death and therefore prevent replication and spread of the pathogen.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised against structural and non structural proteins of any pathogen. The RNase sequence(s) can be substituted against any enzyme sequence that interferes in the pathogen life cycle. Moreover, example 3 can be combined with examples 1-2 and 4-8 in any combination(s).

Example 4

Enhanced coat protein mediated resistance with an antibody-viral coat protein fusion protein

Fusion of a viral coat protein to a recombinant antibody specific for TMV

The steps 1) to 16) of example 1 and/or the steps 1) to 2) of example 2 are repeated with the following adaptations.

1) The transmembrane targeting domain listed in example 1 are removed but the C-terminal anchor and linker sequences of example 2 can be maintained.

2) The N-terminal signal sequence of example 1 is replaced by an upstream located (N-terminal) TMV coat protein monomer and then connected via a flexible linker to the recombinant antibody cDNA.
3) The fusion protein is expressed in the cytosol.
4) Alternatively, the transmembrane domain is replaced by a linker enabling C-terminal fusion with the TMV coat protein monomer. The fusion protein is expressed in the cytosol (without N-terminal signal sequence) or sent into the secretory pathway via a N-terminal signal peptide.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised against structural and non structural proteins of any pathogen. The N-terminal coat protein sequence(s) can be substituted against any sequence (for example Glutathione S-Transferase, Thioredoxin, plant virus movement proteins) that stabilises a cytosolic expressed recombinant antibody and interferes in the pathogen life cycle. Moreover, example 4 can be combined with examples 1-3 and 5-8 in any combination(s).

Example 5

Enhanced resistance by the expression of an anti-viral movement protein antibody

Expression of recombinant antibodies against the TMV 30K movement protein in transgenic tobacco

The steps 1) to 16) of example 1 and/or the steps 1) to 2) of example 2 are repeated with the following adaptations.

1) Specific antibodies recognising the TMV 30K movement protein are raised by hybridoma technology, phage or ribosome display screening and subsequently cloned to form single chain antibodies or any recombinant form thereof.
2) The antibody is expressed in the cytosol or sent into the secretory pathway or membrane localised. The recombinant antibody may cause the desired biological effect without a fusion partner so the toxin sequence may be omitted.
3) For ELISA and surface plasmon resonance the test antigen for antibody function is the native or the recombinant TMV 30K movement protein or domains thereof.

4) Additionally to the bioassays listed in example 1 generated transgenic plants will be tested for broad spectrum resistance against different viral strains or viral genera by inoculation of transgenic plants with virions or infectious transcripts.

Anyone of skill in the art will recognise that these steps can be followed for any other viral pathogen by selecting antibodies or fragments thereof specific for the movement protein and any functional domain. Moreover, example 5 can be combined with examples 1-4 and 6-8 in any combination(s).

Example 6

Enhanced resistance by the expression of antibodies against the Tobacco mosaic virus replicase

Expression of antibodies against the TMV 54K/TMV 183K replicase subunits in transgenic tobacco

The steps 1) to 16) of example 1 and/or the steps 1) to 2) of example 2 are repeated with the following adaptations:

1) Specific antibodies recognising the TMV 54K/183K replicase are raised by hybridoma and phage display or ribosome technology by using recombinant TMV 54K / TMV 183K proteins as the antigen and cloned to form single chain antibody fragments or any recombinant form thereof including bispecific scFvs.
2) These antibodies are expressed in the cytosol or targeted to cytoplasmic face of intracellular membranes, where the virus replication complexes are formed, by using a C-terminal sequence as described in example 2. The recombinant antibody may cause the desired biological effect without a fusion to a toxin.
3) For ELISA and surface plasmon resonance the test antigen for antibody function is the native or the recombinant TMV 54K and 183K replicase proteins or domains thereof.
4) Additionally to the bioassays listed in example 1, generated transgenic plants will be tested for broad spectrum resistance against different viral strains or viral genera by inoculation of transgenic plants with virions or infectious transcripts.

Anyone of skill in the art will recognise that these steps can be followed for any other viral pathogen by selecting antibodies or fragments thereof specific for the movement

protein and any functional domain. Moreover, example 6 can be combined with examples 1-5 and 7-8 in any combination(s).

Example 7

***In vivo* assembly of a molecular pathogenicide**

In vivo assembly of a molecular pathogenicide consisting of a TMV specific antibody labelled with an epitope specific single chain antibody and an epitope tag labelled toxin

The following steps are taken:

1) Antibodies are generated against intact TMV virions and monoclonals are generated by hybridoma technology.
2) Hybridoma cell lines are cloned and cDNA sequences encoding the antibody variable regions are cloned to generate a single chain antibody or any recombinant version thereof binding to the TMV virions (scFv24).
3) The single chain antibody binding to the intact virions (scFv24) is fused to a cloned cDNA from the single chain antibody (scFv-epitag29), which binds to a specific amino acid epitope (epitag29), using a flexible linker such as the linker peptide of *Trichoderma reesi* cellobiohydrolase I (CBHI) to generate a recombinant protein which recognises the pathogen, TMV, and the epitope tag. The scFv-epitag29 has been previously generated (by conventional hybridoma technology and then cloned as an scFv) and the specific epitope identified by phage peptide display. Any other high affinity antibody recognising an identified peptide epitope would be suitable as one half of the binding pair with its corresponding epitope as the other partner.
4) The recombinant gene from step 3 is inserted in a microbial or eukaryotic expression vector.
5) The binding specificity and function (i.e. specificity and affinity) of the recombinant protein from step 3 is checked after expression in a heterologous host, such as in the periplasm of *E.coli*.
6) A signal sequence is added to the N-terminus of the recombinant bispecific scFv construct from step 3, to permit delivery of the protein to the ER and secretion to the apoplast upon expression in plants. A 5' untranslated and a 3'

untranslated region and a detection tag sequence (i.e. c-myc) will be introduced by recombinant DNA technology, if necessary.

7) The chimeric gene from step 6 is inserted into a plant expression vector, e.g. pSS (Voss et al., 1995). Suitable plant expression vectors include suitable promoter, enhancer, terminator and selection marker sequences. In case of markerless and vectorless gene transfer selection marker sequences can be omitted.

8) The cDNA encoding a RIP (ribosome inactivating protein) fused via a suitable linker to the epitag-29 epitope tag (either at the N- or C-terminus), which is specifically recognised by scFv-epitag29, is prepared in parallel to generate a second independent expression construct encoding a tagged RIP gene.

9) The tagged RIP gene is inserted in a microbial or eukaryotic expression vector and the functionality of the RIP-epitope fusion is checked upon expression in a heterologous host.

10) A second, independent plant expression vector, such as pSS, containing the recombinant tagged RIP gene with an N-terminal signal peptide will be prepared, as described in step 6 and step 7. The tagged RIP sequence can then be integrated either in tandem array on the same plasmid as the fusion protein from step 3 or integrated in a second independent plasmid. Note that the sequences remain discrete even if they are in tandem array.

11) Both plant expression constructs listed in steps 6 and 10 are transformed into two independent plant lines or they are co-transformed into the same plant genome, or if the antibody fusion protein from step 3 and the tagged toxin from step 10 are integrated in tandem array that construct is transformed into the same plants.

12) Regenerated plants are screened using the selection marker for integration of the fusion gene in the independent plant lines or the co-transformed lines from step 11.

13) Transgenic plants that express only one of either the antibody fusion protein from step 3 or epitope tagged RIP from step 10 are then sexually crossed to give offspring which will produce both proteins. Plants producing both proteins, whether from this or earlier steps, will produce assembled protein complexes were the two binding partners, the epitope specific antibody (scFv-epitag29)

and the epitope bind and permit assembly of a molecular pathogenicide protein complex.

14) Expression of the bispecific scFv fusion protein and/or the tagged RIP as well as the *in vivo* assembled molecular pathogenicide is monitored by western blotting cell extracts, ELISA or surface plasmon resonance analysis. Activity of the bispecific scFv is checked by ELISA using intact TMV virions as the antigen.

15) Activity of the assembled molecular pathogenicide is checked by ELISA and cytotoxicity assays.

16) Localisation of the fusion protein is checked by indirect immuno-fluorescence, confocal microscopy or immuno-electron microscopy and western blotting or ELISA or surface plasmon resonanace analysis of the intercellular washing fluid.

17) The biological activity of the *in vivo* assembled molecular pathogenicide against TMV is assayed by bioassays on the generated transgenic plants using virions or infectious transcripts.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised and cloned against structural and non structural proteins of any pathogen. Instead of RIPs, similar toxins with cell killing activity can be used. Assembly of molecular pathogenicides can be achieved by using any epitope tag and a given epitope specific antibody with a suitable and stable molecular interaction *in vivo*, or any other pair of proteins that bind to each other. Moreover, example 7 can be combined with examples 1-6 and 8 in any combination(s).

Example 8

In vivo proteolysis

The steps 1) to 16) of example 1 and steps 1) to 2) example 2 are repeated with the following adaptations.

1) A protease cleavage sequence which is processed by a plant and/or a pathogen protease *in vivo* is added either between the recombinant scFv construct and the

C-terminal membrane localisation sequence, using a suitable linker, or between an N-terminal toxin and a C-terminal membrane anchored recombinant antibody.

2) The chimeric gene is inserted into a plant expression vector e.g. pSS (Voss et al., 1995).

3) Suitable protease cleavage sequences include a selected sequence from a random linker library that had been selected by *In vitro* proteolysis and any known protease site that is unique to the fusion protein and does not destroy the molecular viricide and its activity *in vivo*. As an example, the c-myc tag or the CBHI linker is sensitive to plant proteases.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised and cloned against structural and non structural proteins of any pathogen. Moreover, example 8 can be combined with examples 1-7 in any combination(s).

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WE CLAIM

1. A fusion protein comprising
   (a) at least one binding domain specifically recognising an epitope of a plant pathogen; and
   (b) at least one further domain comprising a protein or peptide sequence which is toxic to the pathogen or detrimental to its replication, transmission or life cycle.

2. The fusion protein of claim 1 wherein said domains are linked by covalent or non-covalent bonds.

3. The fusion protein of claim 1 or 2 wherein the toxic activity of the protein or peptide sequence is activated by the presence of the pathogen, a component thereof or a component of a host cell.

4. The fusion protein of claim 3 wherein the toxic activity of the protein or peptide sequence is activated by a pathogen specific or host cell protease.

5. The fusion protein of any one of claims 1 to 4 wherein said binding domain comprises an antibody, a T-cell receptor, a pathogen specific receptor, a peptide specific for an epitope of a pathogen, or at least the binding site of any one of those.

6. The fusion protein of claim 5 wherein said antibody or binding site thereof is a recombinant full-size antibody, dimeric secretory IgA antibody, multimeric IgM antibody, $F(ab')_2$-fragment, Fab-fragment, Fv-fragment, single chain Fv antibody (scFv), bispecific scFv, diabody, single domain antibody (dAb), minibody or molecular recognition unit (MRU), derived from hybridoma cells, synthetic, semi-synthetic, naïve and immunocompetent phage display or ribosome display libraries, or by the generation of fully synthetic designer antibodies.

7. The fusion protein of any one of claims 1 to 6 comprising at least two binding domains for the same or different epitope(s).

8. The fusion protein of claim 7 wherein said epitopes are from the same or different pathogen(s).

9. The fusion protein of any one of claims 1 to 8 wherein the toxin is an enzyme or a viral structural or non-structural protein or a binding domain as defined in any one of claims 1 to 8.

10. The fusion protein of claim 9 wherein said enzyme is chitinase or glucanase, glucose oxidase, superoxide dismutase, DNAse or RNAse or RIP or active fragments thereof either singly or in any combination(s).

11. The fusion protein of any one of claims 1 to 10 wherein the pathogen is a virus, bacterium, mycoplasma, fungus, nematode or insect.

12. The fusion protein of any one of claims 1 to 11 wherein at least one of said domains is fused to a carrier protein.

13. The fusion protein of any one of claims 1 to 12 wherein at least one of said domains comprises a fluorophore.

14. A pathogenicide comprising at least one binding and/or further domain as defined in any one of claims 1 to 13 and a cellular targeting sequence and/or membrane localisation sequence and/or motif that leads to membrane anchoring.

15. The pathogenicide of claim 14 wherein the membrane localisation sequence is proteolytically sensitive.

16. The pathogenicide of claim 14 or 15 wherein said membrane localisation sequence is human T cell receptor transmembrane domains, glyco-

phosphatidyl [redacted]tol (GPI) anchors, KAR1, middle-T antigen or cytochrome b5.

17. The pathogenicide of any one of claims 14 to 16 comprising the fusion protein of any one of claims 1 to 13.

18. The fusion protein of any one of claims 1 to 13 or the pathogenicide of any one of claims 14 to 17 wherein said binding domain(s) and/or said further domain(s) are capable of self assembly in vivo.

19. A polynucleotide encoding the fusion protein of any one of claims 1 to 13 or 18 or the pathogenicide of any one of claims 14 to 18.

20. A vector comprising the polynucleotide of claim 19.

21. A vector comprising separate polynucleotides encoding at least one of said binding domain(s) and/or said further domain(s) of the fusion protein of any one of claims 1 to 13 or 18 or the pathogenicide of any one of claims 14 to 18

22. A composition comprising vectors wherein each vector contains at least one polynucleotide encoding at least one binding domain and/or at least one further domain of the fusion protein of any one of claims 1 to 13 or 18 or the pathogenicide of any one of claims 14 to 18; and wherein the expression of at least two of said polynucleotides results in the production of said fusion protein or said pathogenicide or assembly of the same in vivo.

23. The vector of claim 20 or 21 or the composition of claim 22 wherein the polynucleotide is operatively linked to regulatory sequences allowing the expression of the fusion protein, pathogenicide or the domains thereof in a host cell.

24. The vector or composition of claim 23 wherein said regulatory sequence is a constitutive, chimeric, ubiquitous, tissue specific or inducible promoter.

25. A host cell comprising the polynucleotide of claim 19, the vector of any one of claims 20, 21, 23 or 24, or the composition of any one of claims 22 to 24.

26. A method for the production of a molecular pathogenicide comprising:
(a) culturing the host cell of claim 25 under conditions suitable for the expression of the polynucleotide; and
(b) recovering the fusion protein, pathogenicide or the domains thereof from the culture.

27. A molecular pathogenicide obtainable by the method of claim 26 or encodable by the polynucleotide of claim 19.

28. A method for the production of pathogen resistant transgenic plants, plant cells or plant tissue comprising the introduction of a polynucleotide of claim 19, the vector of claim 20, 21, 23 or 24 or the vectors of the composition of any one of claims 22 to 24 into the genome of a plant, plant cell or plant tissue.

29. A transgenic plant cell which contains stably integrated into the genome a polynucleotide of claim 19, a vector of claim 20, 21, 23 or 24 or the vectors of the composition of any one of claims 22 to 24 or obtainable according to the method of claim 28.

30. A transgenic plant or plant tissue comprising plant cells of claim 29 or obtainable by the method of claim 28.

31. The transgenic plant of claim 30 wherein the fusion protein or pathogenicide are made functional against pathogens by *in vivo* assembly after co-transformation of at least two independent plant expression constructs or after sexual crossing to form hybrid offspring from two parental plants expressing one or more of the domains of the fusion protein or the pathogenicide, or any other form of genetic recombination.

32. The transgenic plant of claim 30 or 31 which displays improved resistance against a patnogen that the wild type plant was susceptible to.

33. Harvestable parts or propagation material of a plant of any one of claims 30 to 32 comprising plant cells of claim 29.

34. A kit comprising the fusion protein of any one of claims 1 to 13 or 18, the pathogenicide of any one of claims 14 to 18, the polynucleotide of claim 19, the vector of claim 20, 21, 23 or 24, the composition of any one of claims 22 to 24 or the molecular pathogenicide of claim 27.

35. Use of the fusion protein of any one of claims 1 to 13 or the pathogenicide of any one of claims 14 to 18, the polynucleotide of claim 19, the vector of claim 20, 21, 23 or 24, the composition of any one of claims 22 to 24 or the molecular pathogenicide of claim 27 for the protection of a plant against the action of a pathogen.

Dated this 16th day of October, 1998

CHANDRAKANT M. JOSHI
AGENT FOR
Fraunhofer Gesellschaft zur Forderung
der angewandten Forschung e.V.

NAME OF APPLICANT: Fraunhofer Gesellschaft zur Forderung - NO. OF SHEETS: 5
: der angewandten Forschung e.V.
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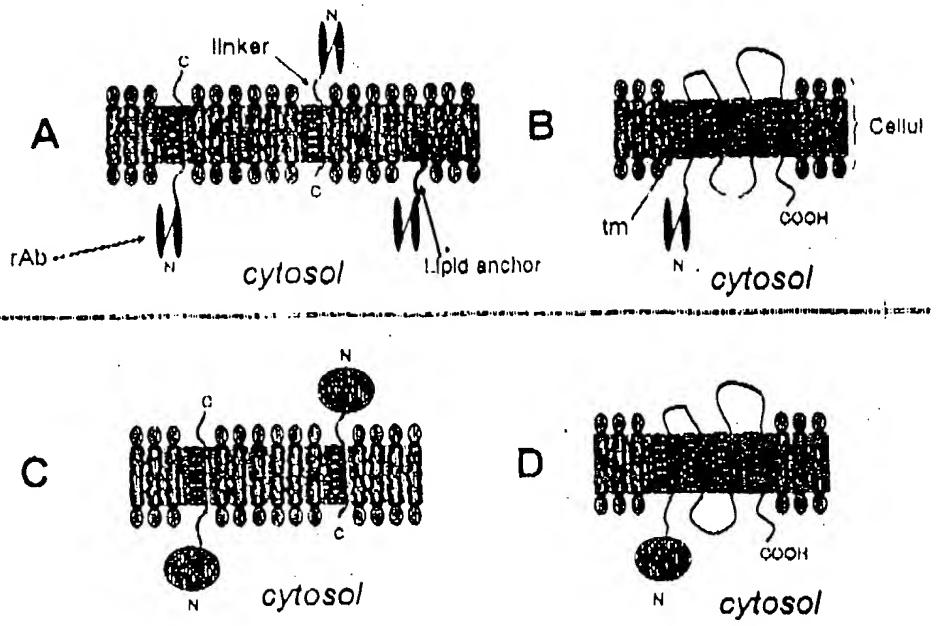

FIG.1

CHANDRAKANT M. JOSHI
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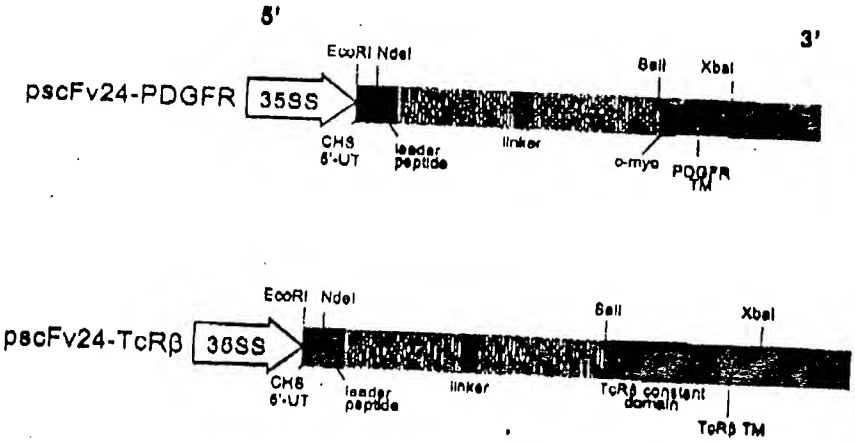

FIG.2

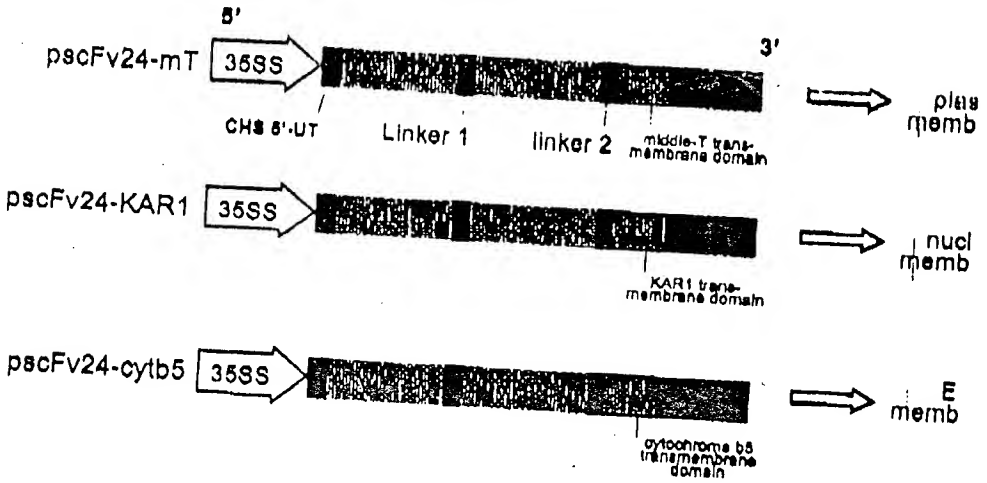

FIG.3

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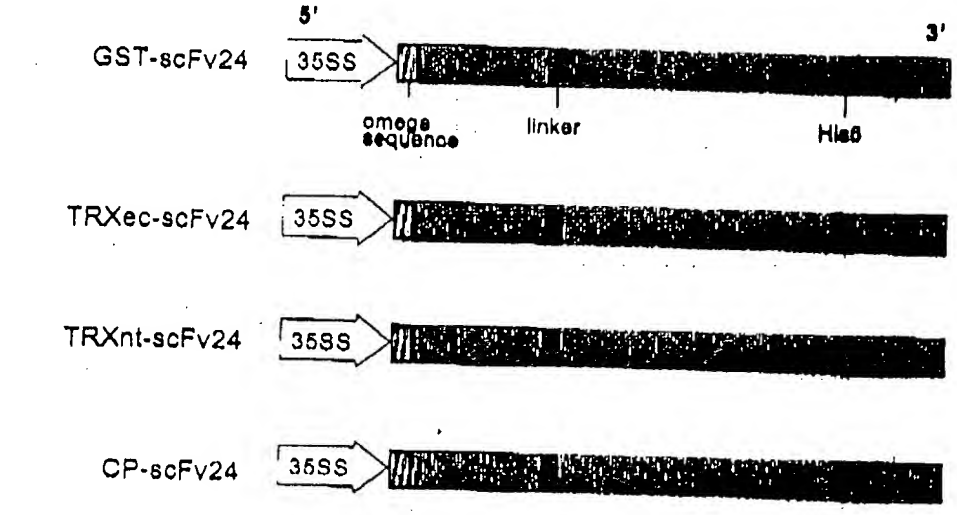
der angewandten Forschung e.V.



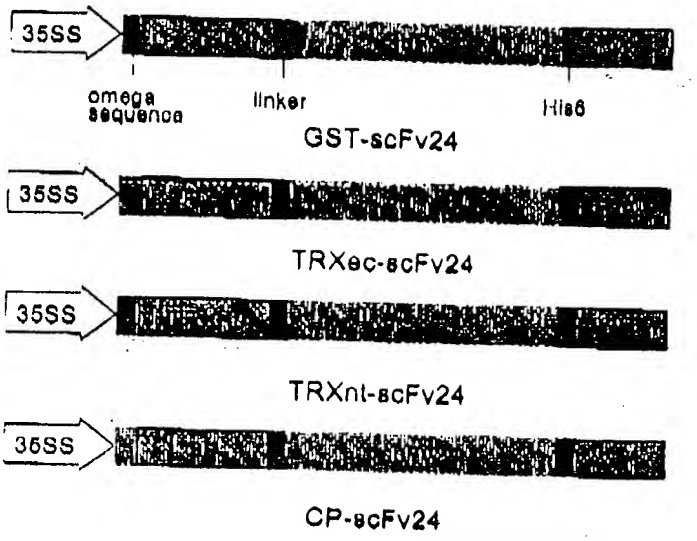


FIG. 4

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NAME OF APPLICANT: Fraunhofer Gesellschaft zur Forderung - NO. OF SHEETS: 5
: der angewandten Forschung e.V.
ALLLICATION NO. : BOM/98 SHEET NO. : 4

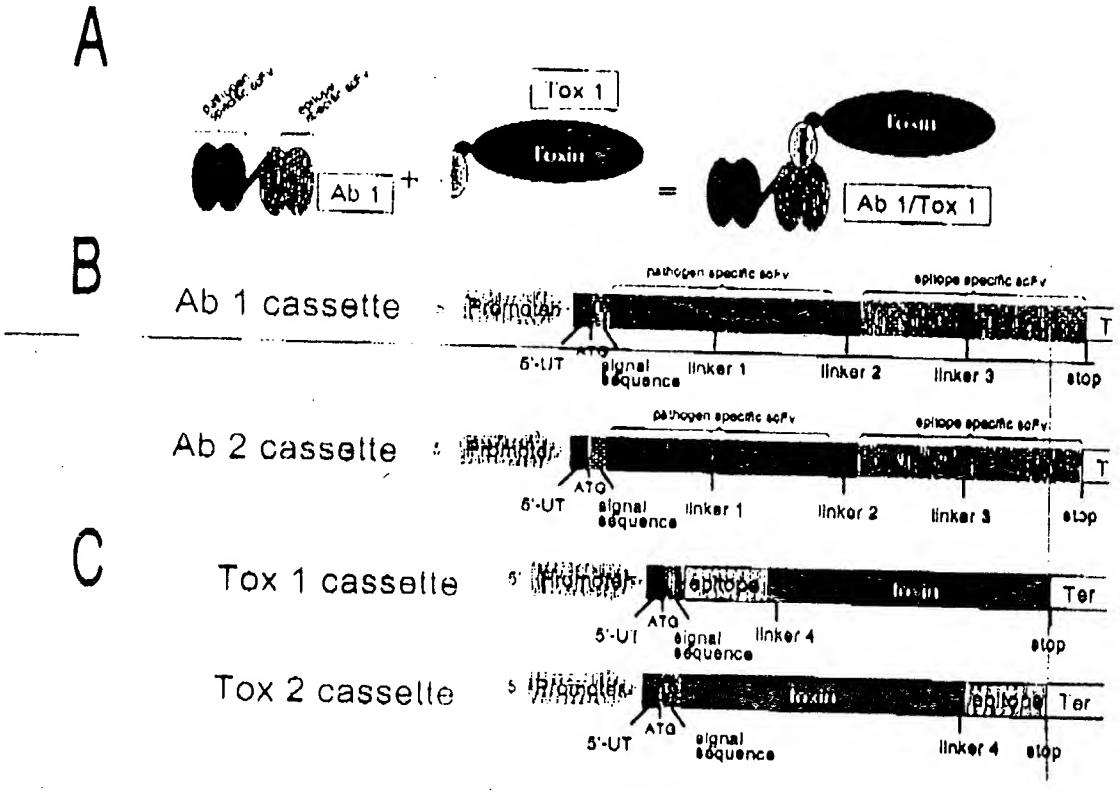

FIG.5

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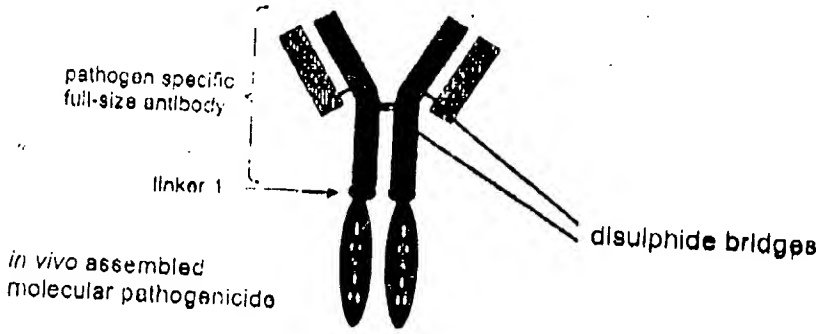
der angewandten Forschung e.V.

NAME OF APPLICANT: Fraunhofer Gesellschaft zur Forderung - NO. OF SHEETS: 5
: der angewandten Forschung e.V.
ALLLICATION NO. : BOM/98 SHEET NO. : 5

A



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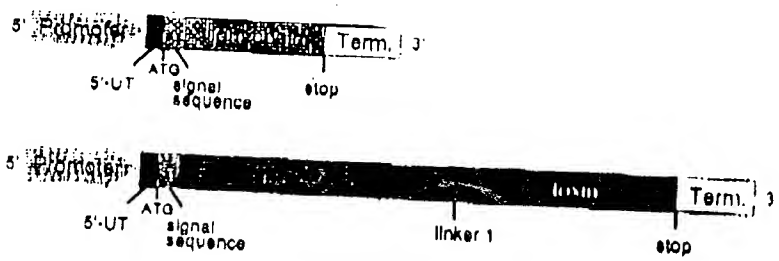

FIG. 6

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FORM - 4

THE PATENTS ACT, 1970.
STATEMENT AND UNDERTAKING UNDER SECTION 8

(see Rule 13)

We FRAUNHOFER GESELLSCHAFT ZUR FORDERUNG DER ANGEWANDTEN FORSCHUNG e. V. of LEONRODSTR. 54, 80636 MUNCHEN, GERMANY, GERMAN Company who have made an application for patent Numbered /BOM/1998 dated / /1998 for our invention relating to MOLECULAR PATHOGENICIDE MEDIATED PLANT DISEASE RESISTANCE hereby declare :-

ORIGINAL

(i) We claim the title to make the said application/deriving the title in the said invention from me/us, have made application for patent for the same/invention in the following countries namely :

(a) NONE

(ii) that the said application has/have been accepted, refused, abondened or withdrawn; NONE

(iii) that the following patent has/have been granted on such application :- NONE

** (iv) that the rights in the application have been assigned to NONE

** (v) that We undertake that upto the date of acceptance of the complete specification filed in connection with our above mentioned application, we would keep the controller informed in writing from time to time of the details regarding application for patents filed outside India from time to time for the same or substantially same invention within three months from date of filing of such application;

** (vi) that the facts and matters stated herein are true to our knowledge, information and belief.

Dated this 16th day of October, 1998.

666 मुंबई BOM 1998

16 OCT 1998

CHANDRAKANT M. JOSHI
AGENT FOR
FRAUNHOFER GESELLSCHAFT ZUR FORDERUNG
DER ANGEWANDTEN FORSCHUNG e. V.

To
The Controller Of Patents,
The Patent Office,
Mumbai
.PA